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The Potential Application and Risks Associated With the Use of Predatory Bacteria as a Biocontrol Agent Against Wound Infections

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14. ABSTRACT

Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of burn, wound, blast and bone infections, with many traditional antimicrobial agents becoming ineffective. Our main hypothesis is that predatory prokaryotes could serve as a novel therapeutic agent to control wound-related bacterial infections. In a previous study, we confirmed that predatory bacteria *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* are able to prey on a wide range of pathogens including bacteria isolated from Wounded Warriors. The aim of this proposal is to address key questions regarding the safety and efficacy of predatory bacteria and investigating predator prey interactions and resistance. Using enrichment culturing techniques we have verified that no genetically stable predation resistant phenotype developed in *K. pneumoniae* and *A. baumannii* host cells following sequential predation by *B. bacteriovorus*. Furthermore, sequential predation by *M. aeruginosavorus* also did not yield host resistance. Our data also confirmed that the predators do not breach their host specificity and attack previously resistant bacteria. Additional enrichment experiments did not produce predators which exhibit enhance predation on a selected host but did yield predators which were acclimated to attack at elevated temperatures.

15. SUBJECT TERMS
Wounded soldiers, Predatory bacteria, Bio-control, Wound infections, Multi-drug-resistant, Bdellovibrio bacteriovorus, Micavibrio aeruginosavorus, Biofilms.

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Introduction

Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of burn, wound, blast and bone infections, while many traditional antimicrobial agents are becoming ineffective. Resistance can be considered as a natural response to the selective pressure of a drug and can develop in both free-floating bacteria, as well as, in surface-attached bacteria or biofilms. One of the major difficulties in controlling surface-attached bacteria is their enhanced resistance to antimicrobial agents i.e. biofilm bacteria can be up to 1000 times more resistant to antimicrobial agents than their planktonic counterparts. Thus, the high doses of antimicrobials required to rid wounds and medical devices of biofilms are impractical. The problem of multidrug-resistant (MDR) bacterial infections in the Wounded Warrior drove researchers to examine other potential anti-bacterial strategies. Among these alternative therapies is the use of biological control agents such as medical maggots, phage, biodebridement, and predatory bacteria.

Our main hypothesis is that predatory prokaryotes are able to serve as a novel topical therapeutic agent in controlling non-treatable, wound-related bacterial infections. In a previous study, we confirmed that predatory bacteria are able to prey on a wide range of pathogens including multidrug resistant bacteria isolated from Wounded Warrior. The aim of this proposal is to address key questions regarding the safety and efficacy of predatory bacteria in *ex vivo* and *in vivo* systems. The proposal is divided into three aims: (I) Investigating predator-prey/host bacteria interactions and resistance, (II) Determining the effect of predatory bacteria on mammalian cells, and (III) Measuring the efficacy of predation and toxicity in animal models.

Body

As stated in the Statement of Work (SOW), the first year is dedicated to Aim-I of the proposed study, Investigating predator-prey/host bacteria interactions and resistance. The goal of this task is to investigate key questions regarding adaptation of the host to the predator. The aim is divided into three subtasks:

Subtask 1.1. Development of genetically stable resistance to predation.
Subtask 1.2. Examine the ability of the predator to breach its host specificity and attack previously resistant bacteria.
Subtask 1.3. Enrich for hyper predatory variants.

Aim I. Investigating predator prey interactions and resistance.
The goal of this task was to investigate key questions regarding adaptation of the host to predation.

Subtask 1.1. Development of genetically stable resistance to predation.
Rationale. It is believed that, unlike antibiotics or phage therapy, the selective pressure of predation does not generate genetically stable resistant variants in the host. Since the appearance of host resistance might reduce the efficacy of predation, we conducted experiments aimed at increasing the selective pressure on the host and assessing if any genetically stable predation resistant phenotypes emerge.

Aim-1, Task-1, Subtask 1.1, Experiment 1. Enriching for host resistant phenotypes by culturing.

In this experiment, host bacteria were cultured with the predator for 24 hrs (predation cycle), thereafter, the remaining host cells were collected by centrifugation, suspended in predator-free media and allowed to grow for an additional 24 hrs (growth cycle). The host cells were collected once more and fresh predators were added (predation cycle). The predation and growth cycle were repeated 20 times. Finally, the reduction in total host was evaluated by CFU enumeration and compared to the initial host reduction measured during the first predation cycle.

Experiment 1.1.1
In these experiments, we have sequentially cultured host bacteria with two predators, *B. bacteriovorus* 109J and *B. bacteriovorus* HD100. The host bacteria used in the experiment was *A. baumannii* NCIMB 12457. Initial predation was determined. Thereafter, host cells were sequentially cultured 20 times. The host population reduction at the final pass was evaluated. All experiments were conducted in triplicates. Data represent the average log change.

Initial predation

Initial reduction (log_{10}) of *A. baumannii* after co-culturing with *B. bacteriovorus* HD100, *B. bacteriovorus* 109J or predator free control.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Initial average log change	+0.2	-2.3	-4.3

Final predation

Final average population reduction (log_{10}) of *A. baumannii* that was sequentially cultured 20 times on ***B. bacteriovorus* HD100**.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	+0.2	-3.6	-3.7

Final average population reduction (log_{10}) of *A. baumannii* that was sequentially cultured 20 times on ***B. bacteriovorus* 109J.**

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	+0.2	-3.8	-3.6

From the data above, it seems that a stable resistant phenotype didn't develop on *A. baumannii* cells which were sequentially cultured on *B. bacteriovorus* 109J or HD100.

** We have initially reported that a stable resistant phenotype did develop on *A. baumannii* cells which were sequentially cultured on *B. bacteriovorus* 109J but not on HD100. However, after closer examination of the data and isolating single colonies we confirmed that the culture was contaminated with a Gram-positive bacterium (confirmed by Gram-staining), which was resistant to predation. The remaining *A. baumannii* isolates did not exhibit predation resistance.

Experiment 1.1.2

In these experiments, we have sequentially cultured host bacteria with two predators, *B. bacteriovorus* 109J and *B. bacteriovorus* HD100. The host bacteria used in this experiment was *K. pneumoniae* ATCC 33495. Initial predation was determined. Thereafter, host cells were sequentially cultured 20 times. The host population reduction at the final pass was evaluated. All experiments were conducted in triplicates. Data represent the average log change. All experiments were conducted in triplicates. Data represent the average log change.

Initial predation

Initial reduction of *K. pneumoniae* (log_{10}) after co-culturing with *B. bacteriovorus* HD100, *B. bacteriovorus* 109J or predator free control.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Initial average log change	+0.1	-5	-2.7

Final predation

Final average population reduction (log_{10}) of *K. pneumoniae* that was sequentially cultured 20 times on ***B. bacteriovorus* HD100**.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	-0.15	-4.4	-3.5

Final average population reduction (log_{10}) of *K. pneumoniae* that was sequentially cultured 20 times on ***B. bacteriovorus* 109J.**

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	+0.1	-5	-2

In order to confirm that genetically stable predation resistant phenotype did not develop, cultures from the final *B. bacteriovorus* 109J enrichment experiment were stored and re-examined in predation experiments.

Reduction of *K. pneumoniae*, that was sequentially cultured 20 times on *B. bacteriovorus* HD100, and stored.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	0	-4.4	-2.7

Reduction of *K. pneumoniae*, that was sequentially cultured 20 times on *B. bacteriovorus* 109J, and stored.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	0	-5.0	-3.9

The data confirms that no stable resistance developed in *K. pneumoniae* following predation.

Summary. The data obtained suggests that no genetically stable predation resistant phenotype developed in *K. pneumoniae* following sequential predation by *B. bacteriovorus* 109J and *B. bacteriovorus* HD100. Furthermore, no genetically stable predation resistant phenotypes developed in *A. baumannii* following sequential predation by *B. bacteriovorus* HD100 and *B. bacteriovorus* 109J.

Aim-1, Task-1, Subtask 1.1, Experiment 1.1.3

In this experiment, we have sequentially cultured host bacteria with *M. aeruginosavorus*. The host bacteria used in this experiment was *P. aeruginosa* Pa14. Initial predation was determined. Thereafter, host cells were sequentially cultured 20 times. The host population reduction at the final pass was evaluated. All experiments were conducted in triplicates. Data represent the average log change.

Initial predation

Initial reduction of *P. aeruginosa* Pa14 after co-culture with *M. aeruginosavorus* or predator free control.

	Control	*M. aeruginosavorus*
Initial average log change	+0.1	-2

Reduction of *P. aeruginosa* Pa14 after 15 cycle of passage with *M. aeruginosavorus* then co-cultured with *M. aeruginosavorus* or predator free control:

	Control	*M. aeruginosavorus*
Average log change	-0.3	-2.3

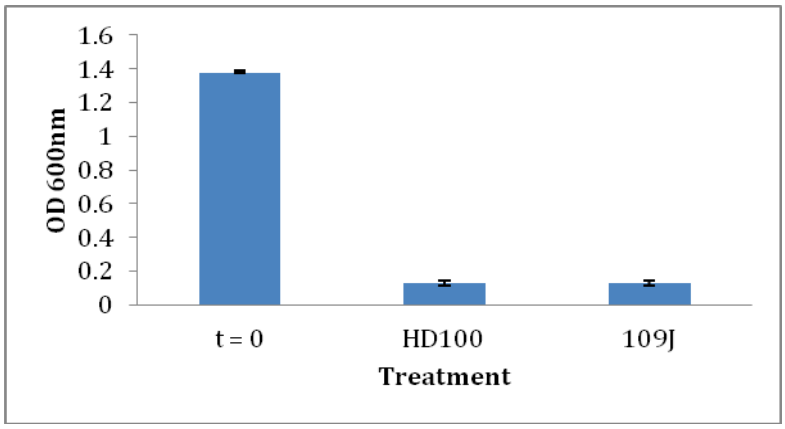
Summary: From the above data it seems *P. aeruginosa* Pa14 did not develop resistance to predation by *M. aeruginosavorus*.

Aim-1, Task-1, Subtask 1.1, Experiment 2. *Enriching for biofilm predation resistant phenotypes.*

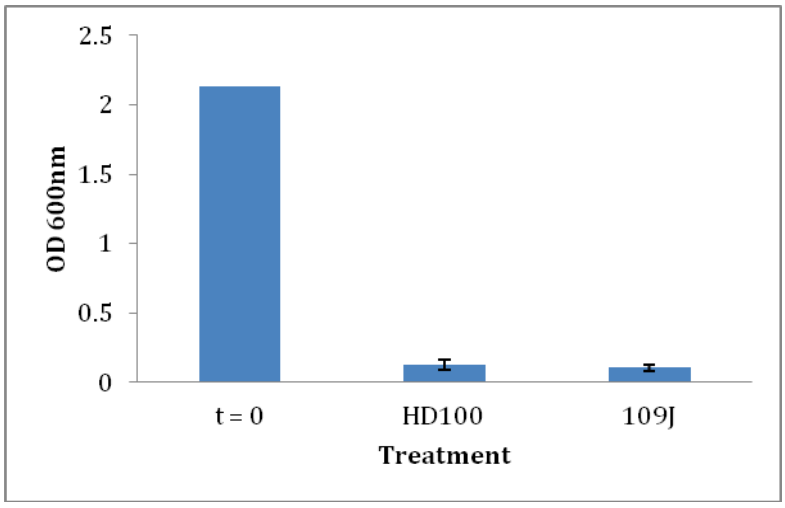
Objective. The aim of this task was to see if biofilm resistant phenotypes develop in biofilm that were cultured multiple times in the presence of the predator.

Bacteria, grown as biofilms, were sequentially grown in the presence of the two predators, *B. bacteriovorus* 109J and predator *B. bacteriovorus* HD100. The host bacteria used in this experiment was *A. baumannii* NCIMB 12457. The experiment was conducted in 8 wells for each treatment and examined for the emergence of a predation resistant biofilm. CV staining was used to quantify biofilm reduction due to predation.

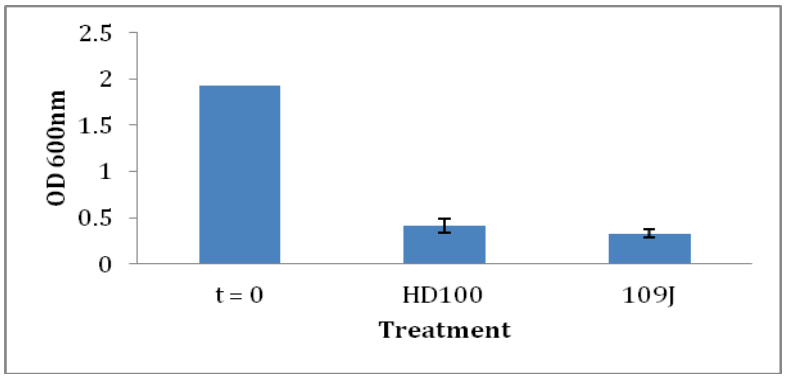
The data below represents the biofilm reduction in host cells in the initial co-culture.



The data below represents the biofilm reduction of host cells that were sequentially cultured 7 times on *B. bacteriovorus* HD100 and then exposed to the predators.



The data below represents the biofilm reduction of host cells that were sequentially cultured 7 times on *B. bacteriovorus* 109J and then exposed to the predators.



Summary. The data obtained suggests that host cells grown as a biofilm do not adapt to form predation resistant biofilms.

Subtask 1.2. Investigating the ability of the predator to breach its host specificity and attack previously resistant bacteria.

Rationale. We have previously conducted experiments aimed at investigating the host range of each predator. However, it could be speculated that during the predation process alterations might cause a change in host specificity. Although a breach in host specificity could be less desirable, as it might allow the predators to attack communal non-pathogenic Gram-negative bacteria, it could be favorably used in order to increase the predatory portfolio of the predator allowing it to attack new desired pathogens. In the following sets of experiments we investigate if a breach or alteration in predator host specificity could arise.

Subtask 1.2. Experiment 1. *Enriching for B. bacteriovorus 109J with altered host specificity.*

The aim of this experiment was to investigate if *B. bacteriovorus* will breach its host specificity and attack a host that initially was resistant to predation. To this end, *B. bacteriovorus* was co-cultured with two hosts *Stenotrophomonas maltophilia* ATCC 13636 and *Streptococcus epidermidis* ATCC 12228. The inability of the predators to prey on the host was confirmed (Table-1 and 3). Thereafter, we have attempted to enrich for *Bdellovibrio* variants that could attack the previously resistant bacteria (Table-2 and 4).

B. bacteriovorus 109J was suspended in a culture containing a 1:1 ratio of the non-host bacteria (*S. maltophilia* or *S. epidermidis*) and the host bacteria *E. coli* strain WM3064, a diaminopimelic acid (DAP) auxotroph. The specific *E. coli* was used in order to allow initial *Bdellovibrio* cell growth. After 48 hrs of predation the *Bdellovibrio* was isolated by filtration (0.45 um filter) and re-cultured in a new co-culture containing a 2:1 ratio of non-host and host (predation resistant bacteria). The predation cycles were repeated 11 times, in each cycle the fraction of the host *E. coli* was reduced. Finally, *Bdellovibrio* cells were isolated and their ability to prey on *S. maltophilia* (Table-1 and 2) or *S. epidermidis* (Table-3 and 4) was examined. All experiments were conducted in triplicates. Data represent the average log change.

Table-1. Initial reduction of *S. maltophilia* after co-culturing with *B. bacteriovorus* 109J.

	Control	*B. bacteriovorus* 109J
Initial average log change	+0.2	-0.2

Table-2. Final population reduction of *S. maltophilia* exposed to predator-free control and the predator *B. bacteriovorus* 109J that was sequentially cultured with *S. maltophilia*.

	Control	*B. bacteriovorus* 109J
Initial average log change	+0.11	+0.11

Table-3. Initial reduction of *S. epidermidis* after co-culturing with *B. bacteriovorus* 109J.

	Control	*B. bacteriovorus* 109J
Final average log change	-0.41	-0.3

Table-4. Final population reduction of *S. epidermidis* exposed to predator-free control and the predator *B. bacteriovorus* 109J that was sequentially cultured with *S. epidermidis*.

	Control	*B. bacteriovorus* 109J
Final average log change	-0.55	-0.55

Summary: The data obtained thus far suggests that *B. bacteriovorus* does not have an ability to breach its host specificity and attack previously resistant bacteria.

Subtask 1.2. Experiment 2. *Enriching for M. aeruginosavorus with altered host specificity.*

The aim of this experiment was to investigate if sequential re-culturing of *M. aeruginosavorus* ARL-13 on a non-host bacteria will result in predation. To this end, *M. aeruginosavorus* was co-cultured with two hosts *Stenotrophomonas maltophilia* ATCC 13636 and *Streptococcus epidermidis* ATCC 12228. The inability of the predators to prey on the host was confirmed (Table-1a and 3a). Thereafter, we have attempted to enrich for *Bdellovibrio* variants that could attack the previously resistant bacteria (Table-2a and 4a).

M. aeruginosavorus was suspended in a culture containing a 1:1 ratio of the non-host bacteria (*S. maltophilia* or *S. epidermidis*) and the host bacteria *E. coli* strain WM3064, a diaminopimelic acid (DAP) auxotroph. The specific *E. coli* was used in order to allow initial *Micavibrio* cell growth. After 48 hrs of predation the *Micavibrio* lysate was re-cultured in a new co-culture containing a 2:1 ratio of non-host and host (predation resistant bacteria). The predation cycles were repeated 11 times, in each cycle the fraction of the host *E. coli* was reduced. Finally, *Micavibrio* cells were isolated and their ability to prey on *S. maltophilia* (Table-1 and 2) or *S. epidermidis* (Table-3 and 4) was examined. All experiments were conducted in triplicates. Data represent the average log change.

Table-1a. Initial reduction of *S. maltophilia* after co-culturing with *M. aeruginosavorus.*

	Control	*M. aeruginosavorus*
Initial average log change	+0.1	+0.1

Table-2a. Final population reduction of *S. maltophilia* exposed to predator-free control and the predator *M. aeruginosavorus* that was sequentially cultured with *S. maltophilia*.

	Control	*M. aeruginosavorus*
Initial average log change	-0.4	-0.4

Table-3a. Initial reduction of *S. epidermidis* after co-culturing with *M. aeruginosavorus.*

	Control	*M. aeruginosavorus*
Final average log change	-0.3	-0.3

Table-4a. Final population reduction of *S. epidermidis* exposed to predator-free control and the predator *M. aeruginosavorus* that was sequentially cultured with *S. epidermidis*.

	Control	*M. aeruginosavorus*
Final average log change	-0.33	-0.2

Summary: The data obtained suggests that *M. aeruginosavorus* does not have an ability to breach its host specificity and attack previously resistant bacteria.

Subtask 1.3. Enriching for hyper predatory variants.
Rationale. As the long-term goal of our study is to develop predatory bacteria as a topical bio-control agent, isolating superior predatory isolates is desired. The aim of this task is to enrich for predator variants that exhibit an elevated predation phenotype.

Experiment 1. *Enriching for hyper virulent B. bacteriovorus.* Previous experiment showed that *B.bacteriovorus* 109J was able to reduce *A. baumannii* strain AB3917 and AB5256 by a **single log,** as opposed to a 5-log reduction seen on most isolates. The aim of this task was to sequentially culture the predator on each of the host in order to enrich for hyper virulent predators.

B. bacteriovorus 109J was co-cultured with *A. baumannii* AB3917 and *A. baumannii* AB5256 for 48 hrs and the reduction of host bacteria was measured (Tables 5 and 5a). After 48 hrs the *Bdellovibrio* was isolated by filtration (0.45um Milex) and re-cultured with fresh *A. baumannii*. As a control, *Bdellovibrio* was cultured with *E. coli*. The predation cycles were repeated 10 times. The predation ability of the culture-enriched *Bdellovibrio* was compared to a control *Bdellovibrio*, which was not co-cultured with *A. baumannii*. All experiments were conducted in triplicates. Data represent the average log change.

Table 5. Initial reduction of *A. baumannii* AB3917 after co-culture with *B. bacteriovorus* 109J or predator free control:

	Control	*B. bacteriovorus* 109J
Initial average log change	-0.3	-5.0

** The outcome of this experiment is somewhat unexpected, as we initially have seen only a one log reduction in the ability of *B. bacteriovorus* 109J to reduce *A. baumannii* AB 3917. We could only speculate that the ability of *B. bacteriovorus* 109J to reduce *A. baumannii* AB 3917 altered during storage of the predator in the lab and continuous passing of the predator.

To measure if we can enrich for hyper virulent *B. bacteriovorus*, the 109J strain was cultured on *A. baumannii* AB3917. As seen in Table 5 a slight increase in the ability of *B. bacteriovorus* to reduce AB3917 was seen.

Table 5a. Final reduction of *A. baumannii* AB3917 after co-culturing with predator free control and *B. bacteriovorus* 109J that was sequentially cultured on AB3917.

	Control	*B. bacteriovorus* 109J
Final average log change	+0.1	-5.5

Enriching for hyper virulent *B. bacteriovorus* on *A. baumannii* AB5256

To measure if we can enrich for hyper virulent *B. bacteriovorus*, the 109J strain was cultured on *A. baumannii* AB5256.

Table 6. Initial reduction of *A. baumannii* AB5256 after co-culturing with *B. bacteriovorus* 109J or predator free control:

	Control	*B. bacteriovorus* 109J
Initial average log change	+0.2	-0.9

Table 6a. Final reduction of *A. baumannii* AB5256 after co-culturing with predator free control and *B. bacteriovorus* 109J that was sequentially cultured with AB5256.

Experiment	Control	*B. bacteriovorus* 109J
Final average log change	-0.1	-0.6

Summary: The data above shows that after continuous predation cycles there were no increase in the ability *B. bacteriovorus* 109J to reduce this specific strain.

Additional experiment.
Since *B. bacteriovorus* 109J that was cultured on *A. baumannii* AB5256 did not become more aggressive, we were interested in measuring if *B. bacteriovorus* 109J that was passed on AB3917 or *E. coli* will be more aggressive against this strain. Predation experiments were performed

using *A. baumannii* AB5256 as host and *B. bacteriovorus* 109J that was passed on AB5256, AB3917 and *E. coli* (Table 7, 7a and 7c respectively) as predator.

Table 7. Reduction of *A. baumannii* AB5256 exposed to predator-free control and the predator *B. bacteriovorus* 109J sequentially cultured with AB5256:

Experiment	Control	*B. bacteriovorus* 109J
Average reduction log change	-0.1	-0.9

Table 7a. Reduction of *A. baumannii* 5256 exposed to predator-free control and the predator *B. bacteriovorus* 109J sequentially cultured with AB3917.

	Control	*B. bacteriovorus* 109J
Average reduction log change	+0.5	-1.2

Table 7c. Reduction of *A. baumannii* 5256 exposed to predator-free control and the predator *B. bacteriovorus* 109J sequentially cultured with *E. coli*.

	Control	*B. bacteriovorus* 109J
Average reduction log change	+0.09	-1.2

Summary: The data obtained suggests that *B. bacteriovorus* 109J could become more virulent on a particular host. However, this increase in predation could develop even in the absence of the prey. Culturing the predator with the prey could slightly enhance predation. On the other hand, increased predation does not develop on all prey with some host bacteria maintaining their tolerance even after continuous culturing. Therefore, we can conclude that enhanced predation is specific and could develop on some host bacteria and not others

Subtask 1.3. Experiment 2. *Enriching for hyper virulent M. aeruginosavorus.* Previous experiments showed that *M. aeruginosavorus* ARL-13 was able to reduce *A. lwoffii* strain ATCC15309 by less than a log. The aim of this task was to sequentially culture the predator on each of the hosts in order to enrich for hyper virulent predators.

M. aeruginosavorus ARL-13 was co-cultured with *A. lwoffii* strain ATCC15309 for 48 hrs and the reduction of host bacteria was measured (Tables 8 and 8a). After 48 hrs the *M. aeruginosavorus* was isolated by filtration (0.45um Milex) and re-cultured with fresh *A. lwoffii*. The predation cycles were repeated 12 times. The predation ability of the culture-enriched *M. aeruginosavorus* was compared to a control *M. aeruginosavorus*.

Table 8. Initial reduction of *A. lwoffii* after co-culturing with *M. aeruginosavorus ARL-13* or predator free control:

	Control	*M. aeruginosavorus*
Initial average reduction log change	+0.2	-0.3

Table 8a. Final reduction of *A. lwoffii* after co-culturing with *M. aeruginosavorus* ARL-13 or predator free control:

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	Control	*M. aeruginosavorus*
Final average reduction log change	0	-0.24

Summary: The data above shows that after continuous predation cycles there were no increase in the ability *M. aeruginosavorus* to reduce *A. lwoffii*.

Experiment 3. *Enriching for predatory bacteria variant more efficient at predation at elevated temperatures.*

The aim of this experiment was to enrich for high temperature predation variants more suitable for medical application. To this end, predation of *Bdellovibrio* and *Micavibrio* at three different temperatures (30°C, 37°C and 39°C) was evaluated.

We observed that predators showed significant host reduction at temperatures 30°C and 37°C but not at 39°C. Hence, enrichment experiment was done using *B. bacteriovorus* 109J lysate and *Micavibrio* lysate from 37°C and then repeated several predation cycles after every 48 hrs until the temperature reached 39°C. In each cycle the incubation temperature was increased by 0.5°C until the temperature reaches 39°C. The predators from the last cycle were isolated and their ability to prey at 39°C was measured.

In this experiment predation was measured by the reduction in culture turbidity using Synergy H1 Hybrid Reader.

The following Table-9 shows the average reduction in culture turbidity. Co-cultures were placed at 39°C, the highest temperature reached during the experiment. The following predators were used:
Sample A: Non temperature acclimated *Bdellovibrio.*
Sample B: Acclimated *Bdellovibrio.*
Sample C: Non temperature acclimated *Micavibrio.*
Sample D: Acclimated *Micavibrio*.

Table 9: Culture turbidity change. Data represent the average of three experiments.

Predator used	Non temperature acclimated *Bdellovibrio*	Acclimated *Bdellovibrio*	Non temperature acclimated *Micavibrio*	Acclimated *Micavibrio*
Average change in culture turbidity	3.6± 3%	43±9%	14±9%	37±5%

Summary: The data obtained suggests that *Bdellovibrio* 109J and *Micavibrio* could be acclimated to prey at an elevated temperature of 39°C.

Additionally we ran the temperature predation experiment with both *Bdellovibrio* 109J and *Micavibrio* using the Synergy H1 Hybrid Reader. *Bdellovibrio* 109J and *Micavibrio lysates were* prepared and incubated in the spectrophotometer at 39°C for 80 hrs. The change in culture turbidity was measured.

The predators used were:
Sample A: Non temperature acclimated *Bdellovibrio.*
Sample B: Acclimated *Bdellovibrio.*
Sample C: Non temperature acclimated *Micavibrio.*
Sample D: Acclimated *Micavibrio*.

Fig 1: *K. pneumoniae* predator-free control incubated at 39°C.

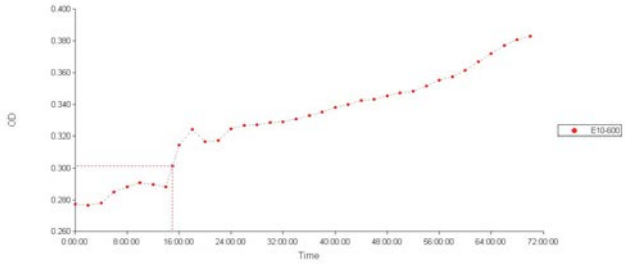


Fig 2: *K. pneumoniae* incubated with a non-temperature acclimated *B. bacteriovorus 109J* set at 39°C.

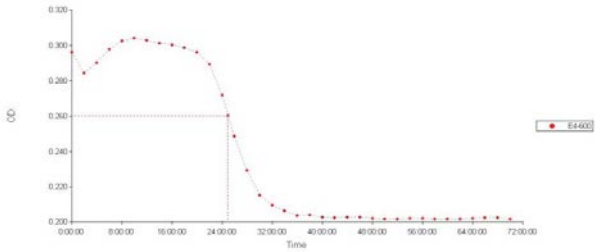


Fig 3: *K. pneumoniae* incubated with a temperature acclimated *B. bacteriovorus 109J* set at 39°C.

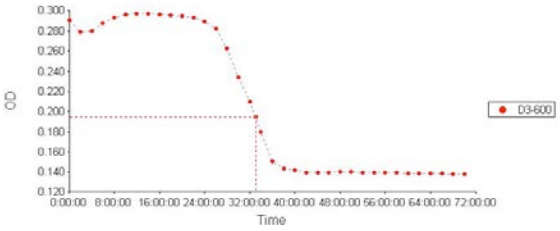


Fig 4: *K. pneumoniae* incubated with a non-temperature acclimated *M. aeruginosavorus* set at 39°C.

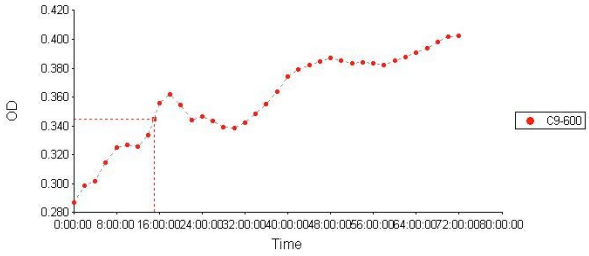
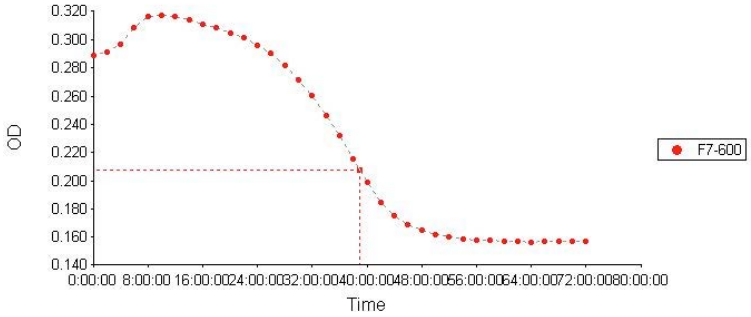


Fig 5: *K. pneumoniae* incubated with a temperature acclimated *M. aeruginosavorus* set at 39°C.



Summary: The data suggests that *B. bacteriovorus* 109J as well as *M. aeruginosavorus* could be acclimated to prey at elevated temperature of 39^{o}C.

Key Research Accomplishments

Aim-1, Subtask 1.1. Development of genetically stable resistance to predation.

- Our data suggest that no genetically stable predation resistant phenotype developed in *K. pneumoniae* and *A. baumannii* following sequential predation by *B. bacteriovorus* 109J and *B. bacteriovorus* HD100.

- Our data suggest that no genetically stable predation resistant phenotype developed in *P. aeruginosa* Pa14 following sequential predation by *M. aeruginosavorus*.

- Our data suggest that host cells grown as a biofilm do not adapt to form predation resistant biofilms.

Aim-1, Subtask 1.2. Investigating the ability of the predator to breach its host specificity and attack previously resistant bacteria.

- Our data suggest that *B. bacteriovorus* does not have an ability to breach its host specificity and attack previously resistant bacteria. This finding was verified using both Gram-negative and Gram-positive host.

- Our data suggest that *M. aeruginosavorus* does not have an ability to breach its host specificity and attack previously resistant bacteria. This finding was verified using both Gram-negative and Gram-positive host.

Subtask 1.3. Enriching for hyper predatory variants.

- Our data suggest that *B. bacteriovorus* 109J could become more virulent on a particular host. However, this increase in predation could develop even in the absence of the prey. Culturing the predator with the prey could slightly enhance predation. On the other hand, increased predation does not develop on all prey with some host bacteria maintaining their tolerance even after continuous culturing. Therefore, we can conclude that enhanced predation is specific and could develop on some host bacteria.

- Our data shows that after continuous predation cycles *M. aeruginosavorus* does not seem to develop an ability to become hyper virulent on *A. lwoffii*.

- Our data suggest that *Bdellovibrio* 109J and *Micavibrio* could be acclimated to prey at an elevated temperature of 39°C.

Reportable Outcome

Manuscripts.

During the lifetime of this proposal we have published two manuscripts. The manuscripts discuss the ability of predatory bacteria to attack defined drug resistant pathogens as well as to treat pathogens associated with eye infection. Although **no** funds from this grant were used in the research leading to the publications, some of the big item equipment purchased through this grant was utilized. The work is also within the scope of our long-term objective of using predator bacteria to treat human infection.

1. **Kadouri, E. D.,** To, K., Shanks, M. Q., and Doi, Y. 2013. Predatory Bacteria; A Potential Ally against Multidrug-Resistant Gram-Negative Pathogens. PLoS ONE. 8(5): e63397. doi:10.1371/journal.pone.0063397.

2. Shanks, M. Q., Davra, R. V., Romanowski, G. E., Brothers, M. K., Stella, A. N., Godboley, D., and **Kadouri. E. D.** 2013. An Eye to a Kill: Using Predatory Bacteria to Control Gram-Negative Pathogens Associated With Ocular Infections. PLoS ONE. PLoS ONE 8(6): e66723. doi:10.1371/journal.pone.0066723.

Oral Presentations.

Several of the findings supported by this grant were presented at the following invited Presentations. (No abstracts were submitted).

1. **Kadouri, D.** Controlling Drug Resistant Bacteria- The Answer is Out There. Department of Oral Biology seminar series. New Jersey Medical School. Newark, NJ. February. 2013.

2. **Kadouri, D.** Controlling Drug Resistant Bacteria- The Answer is Out There. Department of Biochemistry, Microbiology and Immunology. University of Ottawa, Faculty of Medicine. Ontario, Canada. April. 2013.

3. **Kadouri, D**. Bio-control of Drug Resistant Bacteria. Physiology, Ecology and Taxonomy (NEMPET) Meeting. Blue Mountain Lake, NY. June 2013.

Poster Presentations.

1. **Kadouri, E. D.,** and Godboley, D., The use of predatory prokaryotes to control human pathogens and biofilms. 4th ASM Conference on Beneficial Microbes. San Antonio, Texas, October 2012.

Student research opportunities.

This project had given a few students the opportunity to conduct research and gather hands-on scientific experience.

Research Assistant
Somdatta Mukherjee

Rotation Student
Andrew Kim-Rotation M. S. student (GSBS-Rutgers).
Ameet Patheja- Rotation M. S. student (GSBS-Rutgers).
Vanessa Sahs- Rotation M. S. student (GSBS-Rutgers).

Conclusion

Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of burn, wound, blast and bone infections, with many traditional antimicrobial agents becoming ineffective. Our main hypothesis is that predatory prokaryotes could serve as a novel therapeutic agent to control wound-related bacterial infections. In a previous study, we confirmed that predatory bacteria *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* are able to prey on a wide range of pathogens including bacteria isolated from Wounded Warriors. The aim of this proposal is to address key questions regarding the safety and efficacy of predatory bacteria and investigating predator prey interactions and resistance.

It was proposed that, unlike antibiotics or phage therapy, the selective pressure of predation does not generate genetically stable resistant variants in the host. In order to evaluate this hypothesis we conduct experiments aimed at increasing the selective pressure on the host and assessing if any genetically stable predation resistant phenotypes emerge. Using enrichment culturing techniques we have verified that no genetically stable predation resistant phenotype developed in *K. pneumoniae* and *A. baumannii* host cells following sequential predation by *B. bacteriovorus* 109J or HD100. Furthermore, sequential predation by *M. aeruginosavorus* also did not yield resistance in *P. aeruginosa.* As was seen with liquid cultures, *A. baumannii* host cells grown as a biofilm do not adapt to form predation resistant biofilms as a consequence of predation by *B. bacteriovorus* 109J or HD100.

We have previously conducted experiments aimed at investigating the host range of each predator. However, it could be speculated that during the predation process alterations might cause a change in host specificity. A breach in host specificity could be undesirable, as it might allow the predators to attack communal non-pathogenic Gram-negative bacteria. In order to examine if a breach or alteration in predator host specificity could develop, predation resistant bacteria were used, and an attempt was made to enrich for *Bdellovibrio* variants that could attack the previously resistant bacteria. We have used both a Gram-negative and Gram-positive bacteria for this study. The data obtained suggests that *B. bacteriovorus* does not have an ability to breach its host specificity and attack previously resistant bacteria. This was true for both *S. maltophilia* and *S. epidermidis* host cells. We also did not obtain any *M. aeruginosavorus* isolates that breached their host specificity and attacked previously resistant bacteria.

In an attempt to enrich for *B. bacteriovorus* hyper virulent isolates, we found that *B. bacteriovorus* 109J could become more virulent on particular host. However, this increase in predation could develop even in the absence of the prey. Culturing the predator with the prey could slightly enhance predation. On the other hand, increased predation does not develop on all prey with some host bacteria maintaining their tolerance even after continuous culturing. Therefore, we can conclude that enhanced predation is specific and could develop on some host bacteria and not others. Additionally, continuous predation cycles did not seem to increase the ability *M. aeruginosavorus* to reduce a predation tolerant host. Finally, we were able to enrich for predators that were acclimated to attack at elevated temperatures.